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New Standard Guide for Performing the Mouse Lymphoma Assay for Mammalian Cell Mutagenicity

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NEW STANDARD GUIDE FOR PERFORMING THE MOUSE LYMPHOMA ASSAY FOR MAMMALIAN CELL MUTAGENICITY

This guidance document was developed by a joint committee of ASTM and members of the Environmental Mutagen Society (EMS). The EMS panel members were: D. Clive (Chairman), W. Caspari, P. E. Kirby, R. Krehl, M. Moore, J. Novak and T. J. Oberly.

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16. ABSTRACT

The purpose and scope of this standard guide is to present background material and to estalish criteria by which protocols and procedures for conducting the L5178Y/TK$^{+/-}$-3.7.2C mouse lymphoma mutagenicity assay (MLA) can be properly understood and evaluated. These guidelines are also intended to aid researchers and others to gain a better understanding of the critical elements involved with mammalian cell mutagenicity testing. More specifically, this document is intended to provide a guide to researchers that will accomplish the following goals:

- Provide an understanding of the critical procedures (steps) involved in the performance of this mammalian cell mutagenicity test.
- Provide generalized criteria by which researchers can evaluate if they are properly performing, utilizing, and interpreting this assay.
- Provide criteria by which individuals responsible for evaluating mouse lymphoma mutagenicity data can determine if the experiments have been properly performed and interpreted.
- Provide a basis from which new procedures and developments in testing procedures can be evaluated.
- Provide an understanding of the genetic principles involved in the dual mutational endpoint [i.e., gene and chromosomal mutations affecting the thymidine kinase (TK) gene] of this mammalian cell mutagenicity test.

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NEW STANDARD GUIDE FOR PERFORMING THE MOUSE LYMPHOMA ASSAY FOR MAMMALIAN CELL MUTAGENICITY

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A GUIDE FOR PERFORMING THE MOUSE LYMPHOMA ASSAY FOR MAMMALIAN CELL MUTAGENICITY

1. Introduction

This guide was developed by ASTM Sub-committee E47.09 on Genetic Toxicology in order to aid toxicologists, geneticists, biochemists, other researchers and interested persons in the understanding, performance, and analysis of the mammalian cell mutagenicity test that uses the $TK^{+/-}$-3.7.2C strain of L5178Y mouse lymphoma cells. In this rapidly changing area of toxicology, it is not intended for this document to replace, alter, or diminish the usefulness of presently available protocols and procedures.

2. Scope

2.1 The purpose and scope of this standard guide is to present background material and to establish criteria by which protocols and procedures for conducting the L5178Y/$TK^{+/-}$-3.7.2C mouse lymphoma mutagenicity assay (commonly referred to as the mouse lymphoma assay, abbreviated MLA) can be properly understood and evaluated. These guidelines are also intended to aid researchers and others to gain a better understanding of the critical elements involved with mammalian cell mutagenicity testing. More specifically, this document is intended to provide a guide to researchers that will accomplish the following goals:

2.1.1 Provide an understanding of the critical procedures (steps) involved in the performance of this mammalian cell mutagenicity test.

2.1.2 Provide generalized criteria by which researchers can evaluate if they are properly performing, utilizing and interpreting this assay.

2.1.3 Provide criteria by which individuals responsible for evaluating mouse lymphoma mutagenicity data can determine if the experiments have been properly performed and interpreted.

2.1.4 Provide a basis from which new procedures and developments in testing procedures can be evaluated.

2.1.5 Provide an understanding of the genetic principles involved in the dual mutational endpoint [*i.e.*, gene and chromosomal mutations affecting the thymidine kinase (TK) gene] of this mammalian cell mutagenicity test.

3. Significance and Use

This guide is limited to procedures used solely for the testing of substances to determine their mutagenicity and does not apply to other methods and uses such as exploring mechanisms of mutation.

3.1 Recent evidence suggests this assay measures a dual genetic endpoint; therefore, some discussion of the relationships between mammalian cell mutagenicity testing results and the results observed both in pure gene mutational assays and in cytogenetic assays is necessary. However, it is not the intent of this document to discuss other relationships between this mammalian cell mutagenicity testing results and the results observed in other tests for mutagenicity and carcinogenicity.

4. Applicable Documents

4.1 *ASTM Standards:* None known.

5. Description of Terms

5.1 *Mutation:* Any detectable and heritable change in the genetic material not caused by genetic segregation or genetic recombination, which is transmitted to daughter cells. A mutation may be any (or a combination of) detectable, unnatural change affecting the chemical or physical constitution, mutability, replication, phenotypic function, or recombination of one or more deoxyribonucleotides.

5.2 *Gene Mutation:* Any heritable change whose physical extent is restricted to the limits of a single gene.

5.3 *Chromosome Mutation:* A mutation resulting from a structural change to a chromosome involving the gain, loss, or relocation of chromosome segments. Chromosome mutations can be either intrachromosomal or interchromosomal.

5.4 *Clastogen:* Any agent which is capable of inducing chromosome alterations.

5.5 *Mutagen:* Any physical or chemical agent capable of inducing a mutation.

5.6 *Relative Total Growth (RTG):* Used as a means to measure the relative toxicity to cells (survival) following treatment in the mouse lymphoma assay. RTG is calculated according to the method of Clive and Spector **(1)** and includes a measure of growth (relative to the untreated

control) in the suspension phase as well as of the ability to form colonies in the clonal phase of the assay.

6. Test Method

6.1 *Test Principle:* The mouse lymphoma assay utilizes a strain ($TK^{+/-}$-3.7.2C clonal line) of L5178Y mouse lymphoma cells that has been made heterozygous at the thymidine kinase (TK) locus **(1)**. These cells contain thymidine kinase enzyme and are sensitive to the cytostatic and cytotoxic effects of appropriate concentrations of trifluorothymidine (TFT) **(2)**. Forward mutations to the single functional TK gene can result in the loss of thymidine kinase activity and thus the acquisition of TFT-resistance. These mutant cells can be quantitated after an appropriate expression period by cloning in a soft agar medium supplemented with the selective agent, TFT **(2,3)**. A number of protocols have been described **(4-11)**

The assay has been adapted to detect a wide variety of mutagens including those requiring exogenous metabolic activation.

6.2 *Description of Test System:*

6.2.1 *Cell line:* The MLA uses the $TK^{+/-}$ -3.7.2C heterozygote of L5178Y mouse lymphoma cells **(1)**. This cell line has been cytogenetically characterized by banded karyotype at the 230 - 300 band level of resolution **(12,13)**. The chromosome 11 homologs, the known location of the thymidine kinase gene in the mouse **(14)**, have been shown to possess a centromeric heteromorphism which distinguishes the chromosomes 11a and 11b (small and large centromeres, respectively) **(15)**. Through banded karyotype analysis of a large number of $TK^{-/-}$ mutants, this property has allowed the provisional mapping of the single functional TK gene to the terminal two-band region of chromosome 11b in this cell line **(15)**.

6.2.2 *Mutational endpoints:* This cell line forms two classes of TK-deficient (TFT-resistant) mutants based on the criterion of colony size in soft agar cloning medium supplemented with a selective concentration of TFT. Both large and small colony TFT-resistant colonies are totally and heritably TK-deficient by direct enzyme assay **(2,3,5,16,17)**. Further, the majority of small colony TK-deficient ($\sigma TK^{-/-}$) mutants possess chromosome 11b abnormalities ranging from two band insertions or deletions up to whole chromosome translocations **(17,18)**, whereas large colony TK-deficient ($\lambda TK^{-/-}$) mutants appear karyotypically indistinguishable from the parental $TK^{+/-}$ -3.7.2C cells at this same level of resolution. Thus, the mouse lymphoma assay appears to have the dual genetic endpoint of single gene damage and viable chromosomal damage affecting the TK and linked genes and appears to be able to distinguish between

these two types of genetic damage on the basis of mutant colony size.

6.2.3 *Storage:* These cells should be properly stored in liquid nitrogen according to published procedures.

6.2.4 *Integrity of the test system:* There are a number of parameters which can be monitored to assess the integrity of the test system. Each laboratory should establish routine quality control criteria, consistent with the published literature, in order to establish optimum quality of such variables as: water, media components, horse serum, incubator conditions, TFT, agar, plastic or glass cell containers, cell and colony counters, etc. Monitoring the following factors is especially important for the establishment of historical data and ranges in a particular laboratory:

6.2.4.1 *Cell growth rates:* Each laboratory should establish cell growth conditions such that stock cells are maintained in exponential growth with a population doubling time of less than 12 hours. Special attention should be given to the growth rate and general appearance (including microscopic examination) of cell populations following the THMG cleansing procedure. A slight reduction in the growth rate at this time is not unusual but major deviations from the normal range indicate suboptimal health of the cells, a problem with the cleansing medium, or possible mycoplasma contamination.

6.2.4.2 *Plating efficiencies:* Plating efficiency may be a good indicator of the health and vigor of the cells at the end of the expression period, but in general it probably is a better indicator of how well the cloning process and subsequent incubation were controlled. Ideally, absolute plating efficiencies (PE) should be at least 75% but lower efficiencies are acceptable providing the results of the experiment are not compromised. Some factors that may reduce cloning efficiency are: (1) temperature of the cloning medium (CM) when the cells are added; (2) viscosity of CM; (3) pH of CM; (4) improper disaggregation of cell clumps prior to addition to CM; (5) poor control of pH and temperature during the incubation period; (6) insufficient duration of the incubation period; and (7) poor quality of medium components, especially agar **(19)**. Assays with negative results would be considered acceptable if the plating efficiencies of the solvent control cultures are at least 60%. Assays with strong dose-dependent responses may be acceptable with lower cloning efficiencies (*i.e.*, 50-60%), but should be judged on a case-by-case basis; a repeat assay is strongly recommended in such instances. An experiment with a PE below 50% is unacceptable.

6.2.4.3 *Spontaneous mutant frequency:* The spontaneous (background) mutant frequency may vary considerably among laboratories and even within the same laboratory. Each laboratory should use not only published ranges of response but also its own

historical data base with self-imposed limits for determining an acceptable spontaneous mutation frequency. The presence of the metabolic activation system may increase, decrease or leave unaffected the spontaneous mutation frequency. $TK^{+/-}$ cells require periodic cleansing with THMG to select against $TK^{-/-}$ cells (see Section 6.1.4.2) that are accumulating spontaneously in the stock population. Such cleansing on a regular basis will decrease the background mutant frequency and is required to prevent inflated background mutant frequencies.

6.2.4.4 *Positive and negative controls:* Presently, there are no mandatory reference substances for use as concurrent positive and/or negative controls. However, negative controls are usually cultures treated with the solvent used to solubilize and dilute the test compound. Only concentrations of solvent that have no effect on cell growth, cell survival and mutant frequency should be used. Solvents commonly used in the MLA are dimethylsulfoxide (DMSO), saline, water, serum-free medium, ethanol and acetone. These solvents have no detectable effect when applied at concentrations of 1% or less. If alternate solvents are used, sufficient testing should be performed to establish acceptable limits. If a test substance is being tested under metabolic activation conditions, the negative controls should also contain those same activation conditions.

Positive control compounds should be selected for the purpose of detecting any compromise of integrity of the test system. If the test compound is being assessed in the presence and absence of an exogenous metabolic activation system, then a direct-acting mutagen and one requiring metabolic activation must be included in the assay as positive controls. A data base for each positive control compound sufficient to establish upper and lower response limits for the dose(s) used should be established prior to using them to evaluate an assay's acceptability. Assays with negative results having positive control responses outside the acceptable range must be repeated.

6.2.5 *Metabolic activation:* Test compounds should be tested both in the presence and absence of a suitable exogenous mammalian metabolic activation system, to permit the detection of mutagenic metabolites. [An exception *might* be made for testing under only one condition of metabolic activation if the test compound is clearly positive under that condition.] The metabolic activation system usually used in the MLA is Aroclor-induced rat liver S9 plus cofactors. However, the selection of the activating system should be flexible, allowing the investigator to select the system that optimizes the metabolism of the test compound. Therefore, the activation system should be chosen on the basis of its potential for activating the test compound and should be monitored for its ability to induce an acceptable response in the positive controls.

6.2.6 *General testing procedure:* Note: These cells are adapted to suspension growth and should be grown with adequate agitation (to

maintain normal cell doubling times) except after pouring plates for the cloning phase.

Although there are a variety of procedures that can or should be used, the following provides a description of the test as generally run.

6.2.6.1 *Solubility determination:* Prior to the first MLA experiment, the compound's solubility in solvents of choice (see § 6.2.4.4) should be determined. A target stock concentration is 100 times the highest concentration expected to be tested in non-aqueous solvents; lower multiples of the highest expected test concentration are sufficient for saline, water (with due regard for nutrient dilution or hypotonicity) or serum-free medium. In instances of low solubility relative to cytotoxicity, a 1.0X stock solution can be prepared in serum-free medium; this can be added to cells pelletted from their growth medium in their individual tubes.

6.2.6.2 *Dose range:* A preliminary dose range-finding test is usually conducted at concentrations spanning several orders of magnitude, both in the presence and absence of an appropriate exogenous mammalian metabolic activation system. Final testing is conducted in the presence and absence of mammalian metabolic activation at several concentrations based on the findings of the dose range-finding test. These concentrations should include one in which the relative total growth approaches 10% that of solvent controls, where necessary and possible. The highest dose used will depend on the solubility and toxicity of the test compound but should not exceed 10 mg/ml.

Selection of the maximum treatment level should be made on a compound-by-compound basis with special attention to factors such as toxicity, osmolality, trace contaminants and acidity of the test material (S. Galloway, personal communication).

6.2.6.3 *Treatment:* Sufficient numbers of cells should be treated to permit adequate statistical conclusions. Their treatment should be for long enough time and to high enough concentrations of test compound so as to permit adequate assessment of mutagenic risk.

Treatment of 6×10^6 cells with the appropriate concentrations of test compound or substance is typically for 4.0 hours at 37°C. Each series of treatments (*i.e.,* with or without exogenous metabolic activation) consists of at least duplicate solvent controls, appropriate positive control(s) and culture(s) at each dose level. (Note: Many laboratories prefer to dose in duplicate or triplicate.) Following treatment the cells are washed free of test compound or substance by repeated centrifugations and resuspended in fresh culture medium at a cell concentration, usually in the range of $2\text{-}3 \times 10^5$ cells/ml, that is compatible with continued exponential growth. In order to minimize stochastic effects, the total number of mutant cells in each culture should not fall below about 200--*i.e.,* the total number of

cells in each culture should always exceed 5 x 10^6.

6.2.6.4 *Expression:* Each treated and control culture is typically maintained with sufficient agitation for *ca.* 2 days at 37°C with daily cell counts and dilutions to maintain exponential growth and to allow expression of any induced $TK^{-/-}$ mutations **(20)**.

6.2.6.5 *Cloning:* Following expression of induced $TK^{-/-}$ mutations, appropriately selected cultures are cloned in soft agar cloning medium for viable count (VC plates) and for mutant number in the presence of stringent selective concentrations of TFT (TFT plates). Cloned cells are incubated at 5% CO_2, 37°C for 9-12 days to allow colonial growth.

6.2.6.6 *Scoring and sizing colonies:* After incubation each set of VC and TFT plates is scored for number of colonies. In addition, the colonies on the TFT plates can be electronically sized in order to characterize any induced genetic damage as chromosomal or subchromosomal in extent.

7. Test Materials

7.1 *Media:* Fischer **(21)** successfully adapted L5178Y mouse leukemic cells to growth in suspension culture. In developing and validating the L5178Y mouse lymphoma assay, Clive and associates **(4)** routinely used Fischer's medium; however, other laboratories have recently validated the assay with RPMI 1640 medium **(6,7,9)**. Either medium can be used; however, it is important to note several differences between them. The most important of these is the large difference in phosphate concentration, a factor which can affect the stringency of TFT selection **(22)** if proper precautions concerning horse serum heat-inactivation and/or quality are not taken (see Section 7.1.4.1). Secondly, the effective concentrations of cleansing medium components is dependent on the type of base medium used (see Section 7.1.4.2).

7.1.1 *Base medium:* A base medium is generally prepared from powdered formulation or is purchased as a 10X or 1X liquid. Some laboratories prepare 2X medium which can be used for a variety of media preparations. Pluronic F68 (Wyandotte) must be added to the base medium to facilitate growth in suspension culture. Other supplements usually include antibiotics, sodium pyruvate and, occasionally, glutamine Refer to references in § 7.1 for suggested concentrations.

7.1.2 *Growth medium:* Growth medium is prepared by supplementing the base medium with horse serum, usually 10% by volume.

7.1.3 *Cloning medium:* Cloning medium is growth medium further supplemented with agar (Noble, purified or BBL; see ref. **19**) and often with additional serum. Each investigator should determine serum and agar concentrations which yield the best cloning conditions in their laboratory. Refer to references in § 7.1 for agar and serum concentrations as they vary between laboratories. Serum concentration is often adjusted to 20% in the cloning medium since this concentration has been reported to provide the highest cloning efficiency for the L5178Y cells **(23)**; however, this optimum may vary among lots of horse serum and among laboratories.

7.1.4 *Selective media:* There are two types of selective media routinely used in the MLA: cloning medium supplemented with TFT to permit quantitation and characterization of $TK^{-/-}$ mutants; and THMG cleansing medium which keeps the spontaneous $TK^{-/-}$ mutants mutant frequency at a minimum, thereby optimizing the assay sensitivity.

7.1.4.1 *TFT selection:* Cloning medium supplemented with TFT is used to arrest growth of $TK^{+/-}$ cells and to allow clonal growth of $TK^{-/-}$ cells. The optimal concentration of TFT may vary among laboratories, but is usually in the range of 1-5 μg/ml. Those laboratories utilizing RPMI 1640 medium may find it necessary to use a TFT concentration at the higher end of this range. Each laboratory should establish the efficacy of their TFT selection by appropriate means.

Different lots of horse serum vary in their ability to inactivate TFT, possibly resulting from varying amounts of the enzyme thymidine phosphorylase. This enzyme, in the presence of inorganic phosphate, converts TFT to an inactive form. The approximately six-fold higher level of inorganic phosphate present in RPMI 1640 medium (relative to Fischer's medium) drives this inactivation more rapidly in RPMI-based cloning medium if the serum is improperly heat-inactivated, thereby critically decreasing TFT-selection stringency in the mutant selection plates. This can be overridden by a combination of increased TFT concentration and extra attention to the proper heat-inactivation of the horse serum (*i.e.,* insure that the serum reaches 56°C prior to initiating the 30 minute incubation; Novak, personal communication) **(8,24)**. Such precautions are less important if Fischer's medium is used.

7.1.4.2 *THMG cleansing:* Cleansing medium (growth medium supplemented with THMG) is used to rid the stock culture of spontaneously accumulated $TK^{-/-}$ mutants. It is composed of methotrexate (M), to block folate-dependent thymidylate synthetase production of thymidine monophosphate (TMP), thus forcing the cells into dependency on the TK salvage pathway of TMP synthesis; thymidine (T) and hypoxanthine (H), to bypass the folate block in TK-competent cells, and glycine (G) as a methyl group source. In $TK^{-/-}$ mutant cells, the exogenous thymidine cannot be phosphorylated and these cells die from TMP deficiency. Following 24 hrs growth in the cleansing medium, the

stock culture is centrifuged and the cells are washed free of unbound methotrexate and resuspended in growth medium supplemented with THG (*i.e.*, THMG without methotrexate) for one to three days (weekend). This permits the cells to fully recover from the remaining bound methotrexate and resume synthesis of TMP and purines by the folate-dependent pathways. Cells should be allowed to totally recover from the metabolic stress of the cleansing procedure (about two to three days) before being used in a test.

While it has been suggested that the cleansing procedure be performed on a weekly basis, some laboratories may find a less frequent cleansing schedule acceptable, providing a low background mutation frequency is maintained. Other alternatives include: freezing populations of freshly cleansed cells and thawing them a few days prior to use; using cultures grown from a very low inoculum (ca. 600 cells/culture); or maintaining an uncleansed population of cells and cleansing a portion of these cells prior to use. In these cases the exposure of $TK^{+/-}$ cells to methotrexate, which, in the absence of THG is known to induce mutations, can be reduced to a minimum.

For specific concentrations of the ingredients and cell populations used in the cleansing step, refer to references in § 7.1. It is important to note for those laboratories utilizing RPMI 1640 medium that slightly higher concentrations of THMG and THG are required as noted in the literature.

7.1.5 *Quality control of media:* The quality of culture media represents the single most common cause of problems with the MLA. A number of factors are known to contribute to variations in medium quality, the principal one being water quality. A second identified source of assay problems is the lot and source of agar and even the use of a dirty autoclave to sterilize the agar. Serum requires particular precautions with RPMI medium **(8,24)**; see § 7.1.4.1 and § 7.1.4.2. For these reasons, rigorous methods for media quality control should be established for each laboratory which should address the ability to support: (1) suspension growth of both low (≤ 1000 cells/ml) and high (> 1 x 10^6 cells/ml) cell inocula; (2) high cloning efficiencies under non-selective conditions; (3) adequate recoveries of small and large colony $TK^{-/-}$ mutants; and (4) appropriate diameters of non-mutant and both classes of mutant colonies. Each of these quantities should be consistent with published literature values.

7.2 *Metabolic Activation System:* The metabolic activation system may take the form of either whole cells [*e.g.*, co-cultivated rat hepatocytes **(25,26)**] or cell homogenates [*e.g.*, Aroclor-induced rat liver S9 **(5)**; Aroclor-induced hamster or mouse S9 **(27)**].

7.2.1 *Sources:* Preparations designed to provide metabolic activation may be prepared from a variety of sources depending on the needs of a

particular assay. Factors which may vary include, for example, species, sex, tissue, age, method of induction, and method of preparation.

7.2.2 *Cofactor mixes* for enzyme preparations should be shown to support enzyme activity, as measured either directly or by a biological effect.

7.2.3 *Metabolic activities:* The metabolic activation system to be used should be capable of converting appropiate known promutagens to mutagens.

8. Test Data (*First 5 sections as Mayo would have them*)

8.1 *Gathering Data:* Three classes of information can be obtained in the MLA. The first two--cell density measurements during expression and colony counts after the cloning procedure--are necessary for calculating the relative total growth and the mutant frequency resulting from each treatment. The third type of information--the size distribution of mutant colonies within a given treatment--may be useful in classifying the mechanism of action of the mutagen. All data should be recorded wuch that a permanent record of the results is available.

8.1.1 *Daily cell counts:* Cell counts on each culture are obtained on each day following treatment. These counts may be obtained with the use of an automated particle counter or with a hemacytometer. Care should be taken to break up cell clumps prior to sampling for the cell count.

8.1.2 *Counting colonies:* After a suitable incubation period, the Petri dishes are examined and counted. The colonies are best counted with an electronic colony counter. The advantages of using an electronic counter include speed and a direct interface with a computer or data acquisition system. With proper adjustment, electronic counting compares favorably with manual counting without the disadvantage of scorer fatigue. However, one should be sure that no precipitate is in the dishes, especially the mutant dishes, since they receive a larger volume originating from the treatment tube. Also some lots of horse serum can contain particles (*e.g.,* of lipid) which are large enough to be counted by an electronic colony counter. Another potential problem in counting colonies electronically is the phenomenon of monolayer growth which occurs when the occasional colony forms and spreads in a smear in the interface between plate and cloning medium. Although this happens normally due to chance positioning of the cells near this lower boundary of the cloning medium, excessively high numbers of spreading colonies may indicate a problem with the agar concentration or consistency. A careful visual examination of all plates as they are counted will eliminate the possibility of counting artifacts.

8.1.3 *Colony sizing:* Additional information about a chemical's mechanism of action may be obtained by determining the size distribution of mutant colonies. If sizing is to be done, several variables become more important than if only total colony numbers are being counted. All incubator conditions must be closely monitored during the entire incubation period. Fluctuations in CO_2, relative humidity or temperature can retard growth and thus decrease colony sizes. A similar effect can result from nutrient depletion due to overcrowding of colonies. The minimum colony diameter which the electronic colony counter will detect as well as the incubation time period which will yield adequate small colony growth must be established for each laboratory.

8.1.4 *Plotting data (optional):* When test data are clearly negative, graphical representation usually is not useful and ordinarily need not be done, except in cases in which data acquisition and processing is fully automated. Visualization of data in graphical form often aids in their interpretation, so that maual or computer-directed plotting of results is a useful step.

8.1 *Treatment of Results:*

8.1.1 *Total Growth:* Most mammalian cell mutagenicity assays determine survival by cloning shortly after treatment. One difficulty with this is that, for a few hours following treatment by some compounds, some cells cannot survive when sparsely distributed (as in a determination of plating efficiency) but can survive under crowded tissue culture conditions where, for example, metabolic cooperation effects can occur; this results in a spuriously low estimation of survival. Since mutants and mutant progenitors are carried in mass culture, it makes most sense to measure viability under the same conditions.

To accomplish this, a quantity called total growth is measured in the MLA. This amounts to determining, for each cell entering into the treatment, how many progeny are produced, relative to solvent treated controls, by the time that the cells have totally recovered from the treatment. Cells unable to divide a minimum of approximately 10 times will not form detectable colonies, resulting in a decrease in total growth. In addition, viable cells with transiently suppressed growth rate will have a somewhat decreased total growth due to slower growth in suspension culture; these later may form detectable colonies. Thus, in addition to giving a better estimate of viability than plating efficiency, total growth can serve as a sensitive indicator of relevant chemical-biological interactions that plating efficiency determinations would miss.

8.1.2 *Gathering data:* All data should be collected in an orderly fashion to facilitate a rapid and accurate assessment of mutagenicity. Data should be well documented and should include all pertinent information regarding (1) maintenance of cell stock cultures; (2) preparation of all cell cultures for testing; (3) cell counts and manipulations post-exposure (days one and two); (4) suspension growths; (5) individual and mean colony counts; (6) cloning efficiencies; (7) total survivals (or total growths, absolute and relative); (8) mutation frequencies; (9) relative proportions of large and small colony mutant colonies; and (10) any other preferred mutation indices and/or statistical procedures applied. The data should be easily accessible for timeliness in reporting to regulatory agencies, clients, consultants, principal investigators, etc. Depending on the scope of the operation, a laboratory may automate certain aspects of the assay. For example, the use of a Coulter particle counter and electronic colony counter interfaced with a data acquisition system adds to the timeliness and accuracy in the collection and calculation of data.

8.1.2.1 *Daily growths:* Daily cell counts provide valuable information for maintenance and treated cultures. Monitoring daily growths verify that the cells yield normal growth rates. In treated cultures, the presence and extent of cytotoxicity is measured and compared to concurrent positive and negative controls. Furthermore, one segment of total growth (suspension growth) is accomplished. While many laboratories utilize a Coulter counter in assessing daily cell counts, others prefer a light microscope and hemacytometer for cell counting; although this latter procedure is more labor intensive and time consuming, when performed with trypan blue stain a crude measure of cell viability can be obtained.

8.1.2.2 *Counting VC plates:* After a suitable incubation period (see § 6.2.6.5), the Petri dishes are examined and colonies are counted. This can best be done with an electronic colony counter for reasons of speed and possible direct interface with a computer. With proper calibration, electronic colony counter compares favorably with manual counting without the disadvantage of scorer fatigue. Generally, VC plates do not require the sizing of colonies, which are mostly uniformly large. The VC plates measure cloning efficiency (CE) which should fall within certain limits (see § 7.1.5.5). The CE values may be responsible for the rejection of one or more treatments within an experiment or even the entire experiment; therefore, accurate counting and reporting of VC plate counts is mandatory.

8.1.2.3 *Counting mutant colonies:* Similar advantages to using an electronic colony counter hold for counting mutant colonies. Further, important information on the type of genetic damage being induced can often be obtained by sizing the mutant colonies in addition to simply counting them. It is important to determine the limits of the particular

colony counter in counting the very smallest of the small colonies. Most electronic counters do not count all colonies, and no attempt is made to adjust for the varying number (by chemical and dose) of very small colonies that occur on a plate.

8.1.2.4 *Sizing mutant colonies:* Two classes of TK-deficient (TFT-resistant) mutants, based on colony size and relating to the type of genetic damage induced, have been identified in the MLA (see § 6.2.2). Certain compounds such as methyl methanesulfonate and hycanthone produce predominantly small colony mutants; a smaller number of compounds, such as ethyl methanesulfonate, produce predominantly large colony mutants. Most compounds produce a bimodal population of TK-deficient mutant colonies. For these reasons some laboratories may choose to quantitate the frequencies of each of these two types of mutant colonies. It is important that all colonies be scored in the assessment for mutagenicity although, at present, separate enumeration of small and large colony mutants is not universal.

8.1.3 *Entering data:* Daily cell concentration determinations and individual plate counts must be recorded in laboratory notebooks, on tape or disc, so that a permanent record of the results is available. Total growths and mutant frequencies should be calculated for each treated and cloned culture, and concurrent control data should be added to the historical control data base.

8.1.4 *Plotting data (optional):* When test data are clearly negative, graphical representation does not usually provide additional information and ordinarily need not be done (except for the purpose of accumulating historical data, especially in cases in which data acquisition and processing is fully automated). Visualization of data in graphical form often aids in their interpretation, so that manual or computer-directed plotting of results is a useful step.

8.2 *Statistical Evaluation:* Many test results are clearly negative or positive, and statistical analysis is not needed to provide this answer. What is needed by investigators using the test is a consistent and acceptable method for analyzing experiments, especially those with questionable results. The widely accepted "2-fold"or "modified 2-fold"rules compare favorably with certain others (***reference***), but this approach does not take into account the differences in spontaneous backgrounds among various laboratories. Since most dose response curves in the MLA are non-linear, ANOVA based on linear models is inappropriate. Where there is doubt, investigators are encouraged to incorporate the use of an established statistical method into data analyses providing, of course, that the method is appropriate for MLA data and the appropriate statistical assumptions are met.

The majority of test data in the literature has not been evaluated using formal statistics. In general, decisions of "positive" and "negative" are

made on an *ad hoc* basis. A number of reports in the literature rely on the so called "two-fold" rule. This rule states that if a doubling of mutant frequency over background is seen (*e.g.*, mutant frequencies in excess of 120 x 10^{-6} for a concurrent mean background mutant frequency of 60 x 10^{-6}), the chemical is considered mutagenic. The number of doses or replicates is not a factor in this evaluation. However, the mutagenic and cytotoxic responses must be reproducible and dose-related, and it is more convincing if more than one dose is positive by this criterion.

Recently, a number of statistical procedures have been proposed for the MLA **(5,7,28,29,30,31)**, but there is no consensus as to which particular procedure(s) should be used for data evaluation. It is clear, however, that regardless of the statistical evaluation procedure used, the data evaluated must meet a number of criteria, such as: it must be derived from a defined protocol covering a range of doses both with and without metabolic activation; the results must be reproducible; and the anticipated carcinogenicity or non-carcinogenicity of the chemical must not be a consideration in the evaluation.

8.3 *Interpretation of Results:* Before results can be interpreted, a number of experiments must be performed in each laboratory so that quality control criteria can be defined. These quality control criteria may include limits on mutant frequency ranges and cloning efficiency ranges for the solvent and positive controls as well as toxicity limits for the dosed cultures. While general guidelines can be obtained from the literature, these quality control criteria should be determined for each individual laboratory and should be consistent with literature values. Experiments with known mutagens/carcinogens and non-mutagens/ non-carcinogens should be performed in each laboratory to validate response criteria.

After quality control criteria have been applied to a set of data, the results can be evaluated. The methods used must be consistent from experiment-to-experiment and have a justifiable scientific basis. If the two-fold rule is to be applied, then a doubling of the mutant frequency relative to that of the solvent controls should be noted, preferably with two or more treatments. A dose-related response should accompany this and relative total growths should not be less than 10% of the solvent controls. The reproducibility of a particular response can be helpful when analyzing results, especially for compounds eliciting a weak mutagenic response. This may include treating replicate cultures as well as performing replicate experiments. For clearly positive responses, replicate experiments can often be obtained by cloning the appropriate portions of the dose-ranging experiment and comparing those limited results with the results of the subsequent mutagenicity experiment.

An appropriate statistical approach may be helpful in interpreting certain results. Compounds, mixtures, or preparations that produce mutagenic responses that meet the statistical criteria for a positive response may be

considered to be positive under the particular experimental conditions. Statistical criteria should include an analysis of individual doses as well as an analysis of the trend of the dose response curve. Experiments that are positive only after precipitation or under conditions of high osmolarity, low pH, or other extreme condition should be noted. If results show a response of marginal significance at only one concentration, efforts should be made in a repeat trial to select doses around that dose.

Many investigators have evaluated mouse lymphoma data by using the requirement that a 2-fold increase over background must be met before the compound is declared mutagenic. Such an approach is a quite useful approximation; however, in cases of marginal negative/positive responses, it is strongly recommended that (a) repeat experiment(s) be performed and the variability appropriately assessed. Furthermore, all negative and positive controls must fall within historical ranges that were established in that testing laboratory.

8.3.1 *Requirements for declaring a chemical (or test mixture) mutagenic:* After the experiment has been subjected to the quality control criteria, the data over the 10-100% relative total growth range should be evaluated for doses that are positive and for trends in the dose-response. Statistical methods or a "two-fold rule" may be applied to the data.

If, after applying these principles to the test data, the compound cannot be evaluated as positive, then an evaluation of questionable, equivocal, or negative should be applied to the result

8.3.2 *Requirements for declaring a chemical (or test mixture) non-mutagenic:* An experiment can be called negative only if the quality control criteria set up by that laboratory are strictly met. In particular, positive control results must be unambiguous and within historical range of that laboratory's experience. In addition, minimum upper dose criteria must be met, based either on concentration (*e.g.*, 5 or 10 mg/ml) or cytotoxicity (*e.g.*, 10 or 20% relative total growth). Test concentrations should be spaced sufficiently close to preclude missing mutagenic activity on a steep survival curve. Only when these minimal conditions are met can a compound be considered to be non-mutagenic under the experimental conditions to which it was subjected. Experiments that meet the quality control criteria but cannot be evalusted as positive or negative should be deemed to be questionable.

9. Test Report

9.1 *General:* The test report, whether for submission to a journal or other forms for publication in the open literature or for submission to a regulatory agency, should be as complete as possible and should contain: (1) enough detail to insure the reviewer that the test was performed according to accepted procedures, and (2) sufficient data to allow verification of the results. Certain critical elements which should be considered by the investigator when preparing a report are set forth below. Where the needs of the two types of report may differ, this is noted.

9.2 *Title:* The title should be as complete as possible and should accurately reflect the content of the report.

9.3 *Test Agent:* The name used to identify the test agent should be as complete as possible and may include the CAS registry number, if available, and any commonly used synonyms. A representation of the molecular structure may be given. The source and, if appropriate, lot number of the test agent should be supplied. Complete information as to purity should be given; if analysis for purity has been performed, the method used may be referenced. Analyses not performed by the author of the report may also be referenced. Differentiation should be made between technical and reagent grade substances, if appropiate. Test agents used as they were received from the supplier with no further attempt at analysis or chemical identification should be noted. Mixtures should be identified and the percent composition of components should be given, if known or readily available. The solvent used should be identified and the stability of the test agent both in the solvent and on the shelf should be given. Any special procedures used to enhance stability (e.g.,storage in the cold or dark) should be noted. The physical properties of the test agent may be described and the physical state tested may be given. The interval between receipt of the chemical, preparation stock solutions and use in the assay should be stated and storage conditions during this time should be noted. Any other information about the test agent which the author feels would be relevant should be presented. Where the information is not available or is deemed inappropriate by the author, this should be noted, especially for reports which may be presented to a regulatory agency for review and evaluation.

9.4 *Test System:* The test system should be well documented and the cell source identified. Procedures used in maintaining the efficacy of the test system--*i.e.,* screening for Mycoplasma contamination, mutant cleansing (THMG), enzyme analysis, karyotyping, etc.--should be clearly described in the Standard Operating Procedures. Any additional procedures or modifications to the test system must be clearly outlined and explained.

9.5 *Procedures:* When writing for submission to a journal or other "open-literature" publication, the author should bear in mind that the aim of this section is to permit repetition of the test conditions and to insure reproducibility of the reported results. Methods should be reported as concisely as possible with references to well-established procedures; such citations should be to seminal papers, not to secondary sources. In preparing a report for Agency submission, the author should bear in mind that the aim of this section is verification that the test was performed according to acceptable standards; any information relevant to this determination should be presented. In particular, the procedures used should be detailed as completely as possible. When a published guideline (e.g., OECD--Organization for Economic Cooperation and Development) is being followed, this should be clearly stated. Modifications to the guidelines, to the stated protocol or to any relevant laboratory standard operating procedures (SOP) should be documented and the reasons for such deviations should be clearly stated. Relevent SOPs (e.g., for preparation of the metabolic activation system) should be retained by the author and a copy submitted upon request.

9.6 *Experimental Design:* This section should include the number of concentrations tested; the rationale for dose selection; toxicity information, and information on factors which may affect the results. The latter may include, but is not limited to, solvent, pH deviations, duration of treatment, composition of the media, artificial lighting conditions, length of incubation of mutant and viable count plates. The number of replicates, both within an experiment and of the entire experiment, should be indicated. Positive and negative control data should be presented; for submission to a regulatory agency the historical range of mutant frequencies should be given. Details of the metabolic activation system used should be presented. This may include but is not limited to, supplier, biologic source, enzyme inducers, method of preparation, storege conditions, procedures followed during use, composition of the cofactor mix. Again, the author should present any information relevant to evaluation of the study design and interpretation of the results.

9.7 *Results:* Primary data sufficient to verify the results should be presented. If graphic presentations are included in a test report, they should be accompanied by numeric data. Transformed data should not be presented unless the method of transformation is clearly defined and sufficient primary data is presented to permit verification of the transformation. Relative total growths as well as individual VC and TFT plate counts should be presented for each treated culture as well as for solvent and positive control cultures if the data are to be submitted to a regulatory agency.

9.8 *Data Evaluation:* The author should clearly state his data evaluation criteria and should apply appropriate statistical methodology where applicable.

9.9 *Study Acceptability:* For regulatory purposes, the criteria for acceptability of a study should be clearly stated. Any studies which are not acceptable by the author's criteria should be cited and the reason for rejection given.

9.10 *GLPs:* The test report should clearly state if GLPs (Good Laboratory Practices of the FDA) were followed. For regulatory purposes, this should also include a statement of quality assurance and audit procedures and any relevant findings.

9.11 *Conclusion:* The author's conclusion should be clearly and concisely stated.

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